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Short communication

Lysophosphatidylcholine activates caspase-1 in microglia via a novel pathway involving two inflammasomes

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ABSTRACT

Inflammasomes regulate microglial caspase-1 activation and subsequent neuroinflammatory processes in brain pathology. In the present study, we have identified inflammasomes causing caspase-1 activation following stimulation of microglia with lysophosphatidylcholine (LPC), a proinflammatory lipid generated under pathological conditions in the brain. LPC-induced caspase-1 activation in microglia was found to depend on LPS prestimulation, inflammasome NLRP3 and adaptor molecule ASC. Furthermore, knockdown of inflammasome NLRC4 inhibited LPC-stimulated caspase-1 activity in microglia, suggesting the requirement of two inflammasomes for optimal caspase-1 activity.

1. Introduction

Caspase-1 has been considered as a potential therapeutic target in a variety of neurological diseases (Vezzani et al., 2010; Walsh et al., 2014; Song et al., 2017). It converts biologically inactive precursors of the proinflammatory cytokines interleukin (IL)-1β, IL-18 and IL-33 into their mature, biologically active forms, which play a pivotal role in promoting neuroinflammatory processes (Eder, 2009; Garlanda et al., 2013). Mechanisms underlying caspase-1 activation in microglia are not completely understood. Multiple pathways can lead to caspase-1 activation in microglia, while intracellular protein complexes called inflammasomes are major components of the activation cascade of caspase-1 (Walsh et al., 2014; Song et al., 2017).

Lysophosphatidylcholine (LPC) is a proinflammatory lipid, which is released from apoptotic cells or produced under inflammatory or ischemic conditions in the brain as a result of enhanced phospholipase A_2 activity (Farooqui and Horrocks, 2006; Drzazga et al., 2014). LPC can cause microglial recruitment during brain development (Xu et al., 2016) and microglial activation in brain pathology (Inose et al., 2015). We have previously demonstrated that LPC induces caspase-1 activation in microglia (Stock et al., 2006), which depends on Na^+ influx, reactive oxygen species production and intact lipid rafts (Schilling and Eder, 2010, 2011). Here, we have extended our studies and identified inflammasomes regulating LPC-stimulated caspase-1 activity in microglia.

2. Materials and methods

2.1. Materials

The following drugs were used in this study: synthetic L-α-lysophosphatidylcholine (LPC), palmitoyl (16:0); lipopolysaccharide (LPS) from *E. coli* 055:B5 (both from Sigma-Aldrich, Germany). Stock solutions of 30 mM LPC and 1 mg/ml LPS were prepared in H_2O.

2.2. Cells

All experiments were performed on the microglial cell line BV-2. Cells were cultured permanently in DMEM supplemented with 10% FCS and 2 mM L-glutamine as described previously (Stock et al., 2006). Cells were split twice a week, and were plated on glass coverslips at a density of 1×10^5/ml for subsequent experiments. During experiments, cells were maintained in an extracellular solution containing (in mM): NaCl, 130; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; D-glucose, 10 (pH 7.4) as described previously (Stock et al., 2006; Schilling and Eder, 2010, 2011).

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; DAMPs, damage-associated molecular patterns; IL, interleukin; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; NLRC4, nucleotide-binding domain and leucine-rich repeat caspase recruitment domain 4; NLRP3, nucleotide-binding domain and leucine-rich pyrin domain 3; PAMPs, pathogen-associated molecular patterns

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Table 1
Sequences of the siRNAs used for gene silencing.

Target gene (NCBI accession no.)	siRNA sequences (sense)
ASC/Pycard (NM_023258)	5′-GCUGCAAACGACUAAAGAAdTdT-3′
	5′-GCUACUAUCUGGAGUCGUAdTdT-3′
	5′-CAAUGACUGUGCUUAGAGAdTdT-3′
	5′-CCAGGCCUUGAAGGAAAUAdTdT-3′
NLRC4 (NM_001033367)	5′-GGGUGAAGAUAUCGACAUAdTdT-3′
	5′-GGAUGGGAAUGAAGCUCUAdTdT-3′
	5′-GCUUCUCAACAAUCAAGAAdTdT-3′
	5′-CGUAUAAAUUCUUUCAUAAdTdT-3′
NLRP3 (AY355340)	5′-CCUCUUAUUUGGAGAAGAAdTdT-3′
	5′-GGAUGGCUUUGAUGAGCUAdTdT-3′
	5′-GGAGUAUUUCUUUAAGUAUdTdT-3′
	5′-CCACAAUUCAGACCCACAAdTdT-3′
Non-targeting siRNA	5′-UUCUCCGAACGUGUCACGUdTdT-3′

2.3. siRNA-induced knockdown of NLRC4, NLRP3 and ASC

One day before transfection with siRNA, BV-2 microglial cells were seeded in 35 mm petri dishes at a density of 3×10^5/ml and maintained in DMEM supplemented with 10% FCS and 2 mM L-glutamine. The siRNAs for targeting the murine mRNAs of *ASC* (NCBI accession number NM_023258), *NLRC4* (NCBI accession number NM_001033367) and *NLRP3* (NCBI accession number AY355340) were synthesized by Xeragon/Qiagen (Köln, Germany). Sequences of siRNAs are provided in Table 1. A non-targeting (scrambled) siRNA (200 pmol per dish) was used as a negative control. To achieve maximum efficiencies of gene silencing, cells were transfected with a mixture of 4 different siRNAs, each at 50 pmol per dish. For this purpose, siRNAs were diluted at a 1:20 volume ratio in 0.2 ml serum-free DMEM, and the DharmaFECT® transfection reagent (Perbio Science, Bonn, Germany) was diluted 1:100 in 0.2 ml serum-free DMEM. Diluted siRNAs and the transfection reagent were combined and kept at room temperature for 20 min to allow complex formation. In the meantime, the culture medium was removed from cells and replaced with 1.6 ml fresh DMEM/10% FCS. The siRNA transfection mixtures (0.4 ml) were carefully added to the cells, which were subsequently maintained in a cell culture incubator for 24 h.

Detection of *NLRC4* mRNA, *NLRP3* mRNA and *ASC* mRNA, and their downregulation by the corresponding siRNAs were verified by reverse transcription real-time PCR. Total RNA was isolated from cultured cells by the use of TRIzol® reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 µg of total RNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Five percent of the volumes of the reaction products were used for quantitative real-time PCR amplification with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). PCRs were carried out on a GeneAmp5700 thermocycler (PerkinElmer Life Sciences) under the following conditions (45 cycles): DNA denaturation (15 s) at 94 °C, primer annealing (15 s) and extension of double-stranded DNA at 60 °C (60 s), detection of SYBR® Green fluorescence at 77 °C (30 s). PCR primers used for the amplification reactions are listed in Table 2. Threshold cycle (Ct) values for the genes of interest were subtracted from Ct values for *GAPDH* to obtain δCt values. Differences in transcript levels between cells transfected with gene-specific siRNAs and scrambled siRNA, respectively, were calculated as δCt(gene of interest) − δCt(scrambled). Fold increases in mRNA levels were obtained according to $2^{\delta\delta Ct}$ as described in detail previously (Martens et al., 2007). Pretreatment of BV-2 cells with *NLRC4* siRNA, *NLRP3* siRNA or *ASC* siRNA caused specific reduction in the corresponding mRNA levels by 82.4 ± 15.2%, 56.8 ± 17.6% and 78.2 ± 13.4%, respectively (n = 3 experiments in each case), compared to cells that had been incubated with scrambled siRNA.

Table 2
Primer sequences used for reverse transcription (RT) PCR of the indicated genes. F - forward primer, R - reverse primer.

mRNA	NCBI accession no.	Oligonucleotide sequence
GAPDH-F	BC083149	5′-ACGACCCCTTCATTGACCTCA-3′
GAPDH-R		5′-TTTGGCTCCACCCTTCAAGTG-3′
ASC (Pycard)-F	NM_023258	5′-CTATCTGGAGTCGTATGGCTTG-3′
ASC (Pycard)-R		5′-CAGGTCCATCACCAAGTAGGG-3′
NLRC4-F	NM_001033367	5′-CAGGTGGTCTGATTGACAGC-3′
NLRC4-R		5′-AAGCCTGCCGATCAGTTCCT-3′
NLRP3-F	AY355340	5′-GCCTGTTCTTCCAGACTGGTG-3′
NLRP3-R		5′-TCTGGAGGTTGCAGAGCAGGT-3′

2.4. Detection of caspase-1 activity

Activity of caspase-1 was revealed by FAM-YVAD-FMK (1:150; Immunochemistry Technology, Bloomington, USA) using a fluorescence imaging system as described previously (Stock et al., 2006; Schilling and Eder, 2010, 2011). Images of four different visual fields for three independent experiments per condition were collected and analyzed. Fluorescence intensities of all cells were corrected for background fluorescence.

2.5. Statistics

All data are presented as mean values ± standard error of the mean (SEM) and cell numbers are indicated. The statistical significance of differences between experimental groups was evaluated by one-way ANOVA using the SPSS program. Tukey's test was used for post hoc comparison after confirming homogeneity of variances with Levene's test. Data were considered to be statistically significant with $p < 0.05$.

3. Results

3.1. LPC-induced caspase-1 activity requires LPS pretreatment

As demonstrated in Fig. 1A–B, LPC caused substantial increases in caspase-1 activity in microglial cells pretreated with 1 µg/ml LPS for 6 h. In comparison with unstimulated control cells, mean fluorescence intensity of microglial cells stimulated with 30 µM LPC for 1 h was significantly increased to 381.0 ± 7.5% (n = 482 cells of 3 independent experiments; $p < 0.001$). In contrast, without LPS pretreatment, mean fluorescence intensities did not differ markedly between cells maintained either in the presence or absence of 30 µM LPC for 1 h (Fig. 1C–D). Due to the requirement of LPS pretreatment for LPC-induced caspase-1 activity, microglial cells were exposed to 1 µg/ml LPS for 6 h prior to LPC stimulation in all further experiments.

3.2. LPC-induced caspase-1 activity requires NLRC4, NLRP3 and ASC

To further identify mechanisms underlying LPC-stimulated caspase-1 activity, we tested whether siRNA-induced knockdown of the inflammasome NLRC4, the inflammasome NLRP3 and/or the adaptor protein ASC affect caspase-1 activation in LPC-stimulated microglia. As shown in Fig. 2, control experiments revealed that transfection of cells with non-targeting (scrambled) siRNA did not affect LPC-induced caspase-1 activation. Similar to data obtained in microglial cells that had not been exposed to scrambled siRNA (Fig. 1), FAM-YVAD-FMK fluorescence intensity of microglia pretreated with scrambled siRNA for 24 h increased 4-fold (to 384.3 ± 10.0%; n = 555 cells of 3 independent experiments; $p < 0.001$) in response to stimulation of cells with 30 µM LPC (Fig. 2).

Following siRNA-induced knockdown of inflammasomes NLRC4 or NLRP3, caspase-1 activity was significantly inhibited (Fig. 2), indicating the involvement of both inflammasomes in microglial LPC-